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A Karyotype Comparison Between Two Closely Related Species of *Acrostichum*

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ABSTRACT.—*Acrostichum aureum* and *A. danaeifolium* are morphologically similar sympatric species which grow in mangrove communities. To evaluate the cytological differences between these species, their karyotypes were analyzed with conventional staining, triple-staining with chromomycin A_3 (CMA), distamycin A (DA) and DAPI, silver nitrate, and *in situ* hybridization with 45S rDNA as probe. Both species have the same chromosome number ($2n = 60$) with only small differences in chromosome size and morphology. The CMA^+ banding pattern revealed four terminal bands in *A. danaeifolium* and six in *A. aureum*. $DAPI^+$ bands were not found. The maximum number of nucleoli per interphase nucleus and the number of 45S rDNA sites were consistent with the number of CMA^+ bands: four in *A. danaeifolium* and six in *A. aureum*. All meiotically analyzed materials showed 30^{II} with normal chromosome pairing and segregation, except in one plant with a chromosome bridge and fragment in cells of anaphase I and II. It is suggested that sympatry and karyotypic orthoselection have contributed to keep the morphological and karyological similarities in such widespread species.

Pteridaceae is a large and diverse family of homosporous ferns of almost global distribution. This family comprises 32 genera, 22 of which occur in the Americas. The pantropical genus *Acrostichum* includes at least three species: the paleotropical *A. speciosum* Willd., the pantropical *A. aureum* L. and the neotropical *A. danaeifolium* Langsd. & Fisch. (Tryon and Tryon, 1982). The last two species are widely distributed in Brazil, occurring mainly as sympatric members of mangrove communities. *Acrostichum danaeifolium* also may be found isolated on swampy banks far away from the coast.

These two species are morphologically very similar, although there are differences between fertile fronds, petioles, and paraphyses. For example, in *A. aureum* only the distal few pairs of pinnae are fertile, there are abortive pinnae on the petiole, and the paraphyses (*i.e.*, trichomes occurring between sporangia) are globular, whereas in *A. danaeifolium* the pinnae are fertile from the apex to almost the base of the blades, the petioles have no abortive pinnae, and the paraphyses have laterally extended apices (Adams and Tomlinson, 1979; Proctor, 1985). The chromosome numbers of both species are $n = 30$ or $2n = 60$ (Manton and Sledge, 1954; Walker, 1966, 1985; Dujardin and Tilquin, 1971; Lovis, 1977), although polyploids have been reported in two populations of *A. aureum* with $2n = 120$ (Kawakami, 1980, 1982; Roux, 1993) as well as an aneuploid with $2n = 119$ (Nakato, 1996).

The sympatric distribution of *A. aureum* and *A. danaeifolium* suggests that marked genetic differences may maintain reproductive isolation between the species, and mediate against hybridization and polyploidy, which are frequent events in the evolution of pteridophytes, especially in homosporous genera

TABLE 1. List of *Acrostichum* samples analyzed, with provenance, voucher, and chromosome number.

Species	Provenance	Voucher	*n*	*2n*
A. aureum	Cabo de Santo Agostinho, Pernambuco	ABMarcon et al. 208/27444		60
	Ipojuca, Pernambuco	ABMarcon et al. 267/27451	30	60
	Ipojuca, Pernambuco	—	30	
	João Pessoa, Paraíba	Cultivated, immature		60
	Rio Tinto, Paraíba	ABMarcon & GSBaracho 225/27446	30	60
A. danaeifolium	Areia, Paraíba	Cultivated, immature		60
	Bayeux, Paraíba	LPFelix 9367/27454		60
	Cajá, Paraíba	LPFelix 9369/27452		60
	Ipojuca, Pernambuco	Cultivated, immature	30	
	Itamaracá, Pernambuco	—		60
	João Pessoa, Paraíba	Cultivated, immature		60
	Juarez Távora, Paraíba	LPFelix 9368/27453		60
	Paulista, Pernambuco	ABMarcon & GSBaracho 221/25077	30	
	Paulista, Pernambuco	ABMarcon & GSBaracho 223/25078	30	60
	Recife, Pernambuco	Cultivated, immature		60
	Rio Tinto, Paraíba	ABMarcon & GSBaracho 228/27447	30	60
	Utinga, Bahia	ABMarcon et al. 263/27445		60

(Walker, 1984). However, López (1978) observed the occurrence of morphologically intermediate individuals in the Dominican Republic.

In the study reported here, the cytological divergence between *A. danaeifolium* and *A. aureum* was investigated using conventional cytogenetic techniques to analyze chromosome number and morphology, fluorochrome staining to identify heterochromatin blocks, silver nitrate staining to detect the maximum number of nucleoli, and *in situ* hybridization to localize 45S ribosomal DNA (45S rDNA) sites in the genomes of both species.

MATERIALS AND METHODS

Samples and collection sites are given in Table 1. Part of the collected material was cultivated in the experimental garden of the Department of Botany of the Federal University of Pernambuco, Brazil, and another part of the material was stored as dried voucher specimens in the UFP herbarium for posterior identification.

For mitotic analysis, actively growing root-tips were treated with 0.002 M 8-hydroxyquinoline at room temperature for 1 h, followed by 23 h at 6°C, then fixed in Carnoy's solution (ethanol:acetic acid 3:1) for 2–24 h. For meiotic analysis, young sporangia were fixed directly in Carnoy's for 2–24 h at room temperature. All fixed material was stored in a freezer until needed.

Root-tips were washed twice in distilled water for 5 min, after which they were treated with a mixture of 2% cellulase–20% pectinase for 5–6 h at 37°C and hydrolyzed in 5 N HCl for 30 min at room temperature. The root meristem was isolated, mounted on a microscope slide in 45% acetic acid, squashed under a coverslip (subsequently removed by freezing with liquid nitrogen), dried at room temperature, stained with 1% hematoxylin or 2% Giemsa, and mounted in Entellan (Merck), according to Guerra (1999). For meiotic analysis, sporangia were squashed in 2% acetic-carmine and analyzed. Karyotype formulas were based on measurements of the long and short arm lengths of each chromosome performed on photographs of the best metaphase figures. Chromosome nomenclature, based on centromeric index (short arm/total length x 100), followed the system proposed by Levan *et al.* (1964), allowing comparison with results of previous authors.

In preparation for fluorescent CMA/DA/DAPI staining root-tips were washed in distilled water, treated with a mixture of 2% cellulase–20% pectinase for 5–6 h at 37°C, and squashed in 45% acetic acid on a microscope slide. The slides were aged for 3 days at room temperature, stained with 0.5 mg/ml chromomycin A_3 (CMA) for 1 h, counterstained with 0.1 mg/ml distamycin A (DA) for 30 min, and 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 30 min, then mounted in a mixture of glycerol:McIlvaine's buffer (1:1) containing 2.5 mM $MgCl_2$ (following Schweizer and Ambros, 1994). CMA detects heterochromatin blocks rich in guanine and cytosine and DAPI detects blocks rich in adenine and thymine, whereas distamycin A is a non-fluorescent DNA ligant that increases the contrast between CMA and DAPI.

For silver nitrate staining, root-tips were treated and squashed as described for fluorochrome staining. The silver nitrate staining technique was based on Rufas *et al.* (1987). A small drop of silver nitrate (50%, w/v, in formalin-water) was placed over the squashed cells, covered with a coverslip, and incubated at 60°C in a moist chamber for ca. 10 min.

An rDNA probe isolated from *Arabidopsis thaliana* (SK18S + SK25S) containing two separately recloned fragments of the 45S rDNA repeat, representing the 18S and 25S rDNA (Unfried *et al.*, 1989; Unfried and Gruendler, 1990), kindly supplied by Prof. D. Schweizer, University of Vienna, was marked with biotin-11-dUTP (Sigma, USA) by nick translation. The 5S rDNA probe was obtained from genomic DNA of *Passiflora edulis* by PCR using the primers 5′-GTG CGA TCA TAC CAG C(A/G)(G/T)TAA TGC ACC GG-3′ and 5′-GAG GTG CAA CAC GAG GAC TTC CCA GGA GG-3′ (Gottlob-McHugh *et al.*, 1990). The technique was based on Moscone *et al.* (1996). The probes were added at a final concentration of 1.2–3.0 ng/μl to a hybridization mixture containing 60% (v/v) formamide, 5% (w/v) dextran sulphate, and 0.1 μg/μl salmon sperm in 2xSSC. The hybridization mixture and the cytological preparations were denatured at 75°C for 10 min and hybridized for 18–20 h at 37°C in a moist chamber. The 45S rDNA probe was detected with mouse anti-biotin monoclonal antibody (Dakopatts n° M743) and visualized with rabbit anti-mouse antibody conjugated to tetramethyl rhodamine isothiocyanate (TRITC) (Dakopatts n° R270). The 5S rDNA probe was detected with sheep

anti-digoxigenin antibody conjugated to fluorescein isothiocyanate (FITC) (Boehringer Mannheim n° 1207741) and FITC-conjugated rabbit anti-sheep (Dakopatts F135, DAKO). The slides were stained with 2 μg/ml DAPI, washed in 2xSSC, and mounted in Vectashield H-1000 (Vector Labs).

The slides were examined using a Leica DMLB microscope and the best cells photographed on Kodak ASA 25 Imagelink HQ film for bright-field and Kodak ASA 400 T-MAX film for fluorescence images. Prints were made on Kodak Kodabromide F3.

Results

The chromosome number observed was $2n = 60$ in all the individuals of the two species. The chromosome size and morphology were similar for both species (Fig. 1a, b). The haploid chromosome complement was formed by 1m + 2sm + 19st + 8t in *A. aureum* and 1m + 3sm + 18st + 8t in *A. danaeifolium* (Table 2). The metacentric pair was the second smallest of the complement in *A. aureum*, but in *A. danaeifolium* it was the fifth smallest pair. However, differences in chromosome length between chromosomes of a complement, which determine the ordering, were very small. Satellites were observed in two subtelocentric pairs in both species.The chromosome size exhibited a gradual variation within each complement, ranging from 4.91 to 8.09 μm in *A. aureum* and from 5.06 to 8.04 μm in *A. danaeifolium*. The average chromosome sizes were 6.35 and 6.40 μm and the length of haploid complements was 190.57 and 192.12 μm for *A. aureum* and *A. danaeifolium*, respectively (Table 2).

Silver nitrate staining did not allow the visualization of the nucleolus organizer regions (NORs), although the nucleoli were well defined. In 887 nuclei analyzed of *A. danaeifolium*, the number of nucleoli varied from one to four, with three being the most common (56.3%). Cells with three or four nucleoli generally exhibited one nucleolus much smaller than the others. In *A. aureum* 318 nuclei were analyzed, and the number of nucleoli varied between one and six (Fig. 1c), with four being the most common (39.6%).

Meiotic analysis was performed in three individuals of *A. aureum* and four of *A. danaeifolium*, of which two from each species were growing together (Table 1). Both species nearly always showed normal meiosis, with 30 bivalents (Fig. 1d, e). In a single plant from a population of *A. aureum* some meiocytes showed anaphase I and II with a chromosome bridge and fragment (Fig. 1f).

After CMA/DA/DAPI staining, *A. danaeifolium* exhibited two pairs of subtelocentric chromosomes with a CMA^+ band on their short arms, slightly different in size and brightness (Fig. 2a). The same cell stained with DAPI displayed a homogeneous staining, except the CMA^+ regions, which became negatively stained. For *A. aureum*, three chromosome pairs showed a $CMA^+/DAPI^-$ band, two of them on the short arms of a subtelocentric and a telocentric chromosome pairs and one on the long arms of a subtelocentric pair (Fig. 2c). The band of the short arms of the subtelocentric chromosome pair was smaller

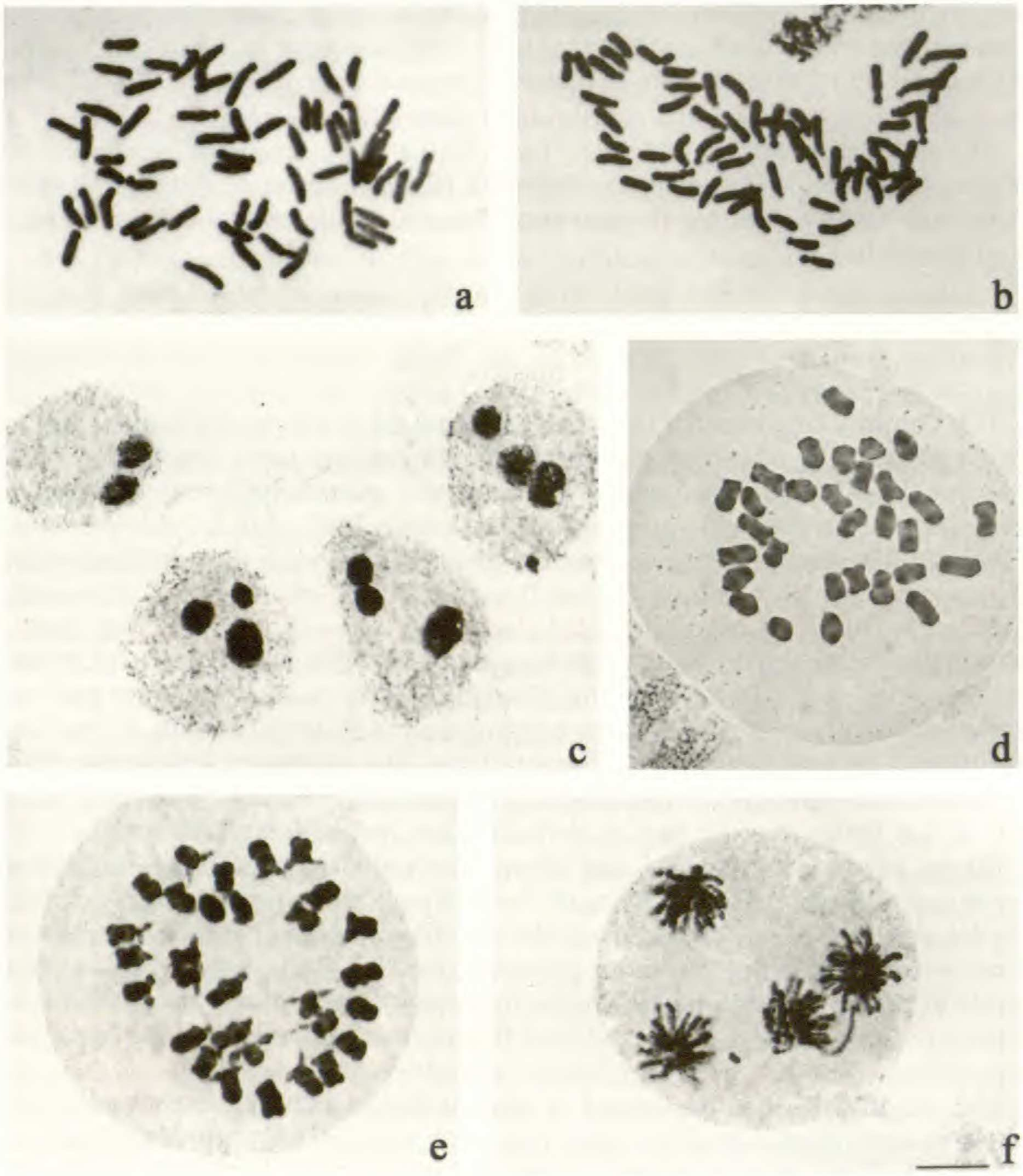

FIG. 1. Mitotic metaphase, nucleolus number, and meiotic behaviour in *Acrostichum*. a, b. Giemsa-stained mitotic metaphase of *A. danaeifolium* (a) and *A. aureum* (b). c. Silver-stained nuclei with 2–4 nucleoli in *A. danaeifolium*. d, e, f. Carmin-stained meiocytes with 30 bivalents in *A. danaeifolium* (d) and *A. aureum* (e) and a chromosome bridge with fragment in a later anaphase II of *A. aureum* (f). Bar represents 10 μm. Note in e the achiasmatic short arms of some acrocentric chromosomes.

and sometimes unstable. No DAPI$^+$ bands were seen on the chromosomes of either of the species (Fig. 2b, d).

In situ hybridization with 45S rDNA fragments labeled the terminal regions of the short arms of two subtelocentric chromosome pairs of *A. danaeifolium*,

TABLE 2. Comparison between chromosome pairs of *Acrostichum aureum* and *A. danaeifolium*, ordered from the largest to the smallest. s, short arm; l, long arm; t, total length; ci, centromeric index (short arm/total length × 100); T, telomeric, ST, subtelomeric; SM, submetacentric; M, metacentric.

	A. aureum				***A. danaeifolium***		
Pair	Measurements (l + s = t)	ci	Type	Pair	Measurements (l + s = t)	ci	Type
1	7.19 + 0.90 = 8.09	11.12	T	1	6.80 + 1.24 = 8.04	15.42	ST
2	6.59 + 1.01 = 7.60	13.29	ST	2	6.37 + 1.53 = 7.90	19.37	ST
3	5.06 + 2.42 = 7.48	32.35	SM	3	7.27 + 0.62 = 7.89	7.86	T
4	6.31 + 0.90 = 7.21	12.48	ST	4	4.89 + 2.77 = 7.66	36.16	SM
5	5.83 + 1.27 = 7.10	17.89	ST	5	5.53 + 1.78 = 7.31	24.35	ST
6	5.69 + 1.38 = 7.07	19.52	ST	6	5.76 + 1.45 = 7.21	20.11	ST
7	5.58 + 1.46 = 7.04	20.74	ST	7	6.99 + 0.00 = 6.99	0.00	T
8	6.22 + 0.74 = 6.96	10.63	T	8	5.34 + 1.59 = 6.93	22.94	ST
9	6.31 + 0.55 = 6.86	8.02	T	9	5.77 + 1.08 = 6.85	15.77	ST
10	5.76 + 1.08 = 6.84	15.78	ST	10	5.06 + 1.57 = 6.63	23.68	ST
11	6.13 + 0.55 = 6.68	8.23	T	11	5.41 + 1.15 = 6.56	17.53	ST
12	5.62 + 0.97 = 6.59	14.72	ST	12	5.62 + 0.90 = 6.52	13.80	ST
13	5.41 + 1.11 = 6.52	17.02	ST	13	5.02 + 1.45 = 6.47	22.41	ST
14	5.06 + 1.38 = 6.44	21.43	ST	14	4.58 + 1.80 = 6.38	28.21	SM
15	4.99 + 1.43 = 6.42	22.27	ST	15	5.00 + 1.35 = 6.35	21.26	ST
16	5.83 + 0.48 = 6.31	7.61	T	16	5.16 + 1.18 = 6.34	18.61	ST
17	5.37 + 0.83 = 6.20	13.39	ST	17	5.20 + 1.11 = 6.31	17.59	ST
18	5.20 + 0.98 = 6.18	15.86	ST	18	6.24 + 0.00 = 6.24	0.00	T
19	5.13 + 0.97 = 6.10	15.90	ST	19	6.13 + 0.00 = 6.13	0.00	T
20	5.41 + 0.62 = 6.03	10.28	T	20	4.79 + 1.30 = 6.09	21.35	ST
21	4.79 + 1.18 = 5.97	19.76	ST	21	5.27 + 0.69 = 5.96	11.58	T
22	4.91 + 0.97 = 5.88	16.50	ST	22	4.37 + 1.59 = 5.96	26.68	SM
23	5.13 + 0.55 = 5.68	9.68	T	23	4.72 + 1.13 = 5.85	19.32	ST
24	4.16 + 1.45 = 5.61	25.85	SM	24	5.76 + 0.00 = 5.76	0.00	T
25	4.94 + 0.76 = 5.70	13.33	ST	25	5.09 + 0.55 = 5.64	9.75	T
26	4.65 + 0.90 = 5.55	16.22	ST	26	3.19 + 2.20 = 5.39	40.82	M
27	5.42 + 0.00 = 5.42	0.00	T	27	4.44 + 0.90 = 5.34	16.85	ST
28	4.17 + 0.83 = 5.00	16.60	ST	28	4.37 + 0.83 = 5.20	15.96	ST
29	3.05 + 2.08 = 5.13	40.54	M	29	4.47 + 0.69 = 5.16	13.37	ST
30	4.22 + 0.69 = 4.91	14.05	ST	30	5.06 + 0.00 = 5.06	0.00	T

with sites slightly different in size (Fig. 2e). In *A. aureum*, there were two sites on the short arms of a subtelocentric and a telocentric chromosome pairs and one on the long arms of a subtelocentric chromosome pair. The site on the telocentric chromosome pair was the smallest, while the other two were of similar size (Fig. 2f). The 5S rDNA probe did not produce any single signal, in spite of repeated attempts.

DISCUSSION

Acrostichum danaeifolium and *A. aureum* are considered diploid taxa with a chromosome base number $x = 30$ (Lovis, 1977). The chromosome number found in *Acrostichum* populations from Northeast Brazil agreed with those

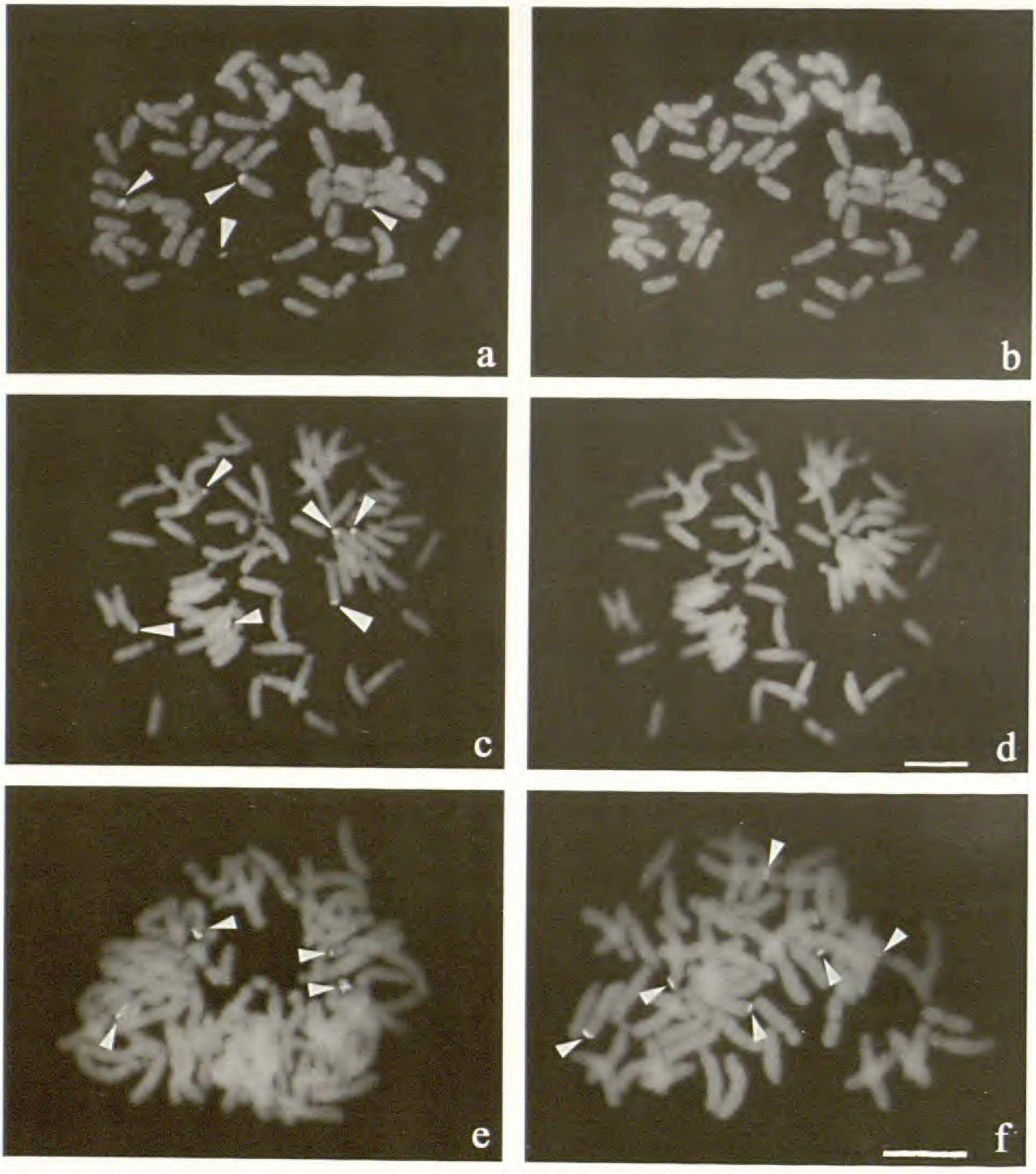

FIG. 2. CMA/DAPI bands and 45S rDNA sites in *Acrostichum danaeifolium* (a, c, e) and *A. aureum* (b, d, f). Arrows in a, c point to CMA^+ bands that are negatively stained with DAPI (b, d). Arrows in e, f indicate rDNA sites. Bars represent 10 μm. CMA/DAPI photographs (a, b, c, d) are at a different magnification in relation to FISH photographs (e, f).

reported for Sri Lankan (Manton and Sledge, 1954), Jamaican, and Trinidadian (Walker, 1966, 1985) populations, with $2n = 60$ and $n = 30$ for both species. Dujardin and Tilquin (1971) also reported $n = 30$ for a sample of *A. aureum* from Congo. On the other hand, Kawakami (1980, 1982) and Roux (1993) reported $2n = 120$ for *A. aureum* from the Japanese island of Iriomote and from Natal (South Africa), respectively, and Nakato (1996) found an individual of

A. aureum from the Iriomote population with $2n = 119$. The latter is the only report of aneuploidy in *Acrostichum*, although dysploids are known in some other pteridophytes (Walker, 1984, 1985).

The karyotypes of *A. aureum* and *A. danaeifolium* were similar in total haploid length (ca. 192 μm), general symmetry, and chromosome size variation. They differed slightly in the karyotype formula: 1m + 2sm + 19st + 8t for *A. aureum* and 1m + 3sm + 18st + 8t for *A. danaeifolium*. The average chromosome size of the tetraploid *A. aureum*, described by Kawakami (1980), was 4.92 μm, therefore, much shorter than the average chromosome size in the present diploid sample (6.35 μm). Although this observed difference may be due to differential chromosome condensation, it most likely is due to chromosome size reduction observed in most polyploids (Raina *et al.*, 1994; Leitch and Bennett, 1997).

In both *Acrostichum* species, the maximum number of nucleoli and CMA^+ blocks were clearly correlated. For *A. danaeifolium*, there were four CMA^+ bands in the metaphase chromosomes and interphase nuclei and up to four nucleoli per nucleus. A similar correlation occurred in *A. aureum*, with six CMA^+ bands and a maximum of six nucleoli per nucleus. The large number of nuclei with a lower number of nucleoli may be related to the tendency of nucleoli to fuse (see, *e.g.*, Moscone *et al.*, 1995). In angiosperms, most nucleolus organizer regions (NORs) are $CMA^+/DAPI^-$ bands (Guerra, 2000), and the same seems to be true in *Acrostichum*. For other pteridophytes, apparently no previous karyological studies have been published using CMA/DAPI or silver nitrate staining.

In angiosperms, NORs are also correlated in number and size with secondary constrictions and sites of 45S rRNA genes. In pteridophytes, sites for 45S rRNA genes have been previously reported only in *Osmunda japonica* Thunb. (Kawakami *et al.*, 1999) and in *Ceratopteris richardii Brongn.* (McGrath and Hickok, 1999), without indication about NORs or secondary constrictions. In *A. danaeifolium*, there were four secondary constrictions and four 45S rDNA sites; in *A. aureum* there also were four secondary constrictions but six 45S rDNA sites. This difference is probably due to the fact that 45S rDNA sites of a cell are not always active in *A. aureum* and some sites may be only rarely activated, resulting in a variable number of secondary constrictions. For example, *Citrus sinensis* (L.) Osbeck has three 45S rDNA sites and a maximum of three nucleoli per cell, but only 2.5% of the metaphases exhibit three secondary constrictions (Pedrosa *et al.*, 1997, 2000).

The meiotic analysis of both species of *Acrostichum* did not show any significant variation, even in sympatric populations. The anaphase bridge observed in a single individual of *A. aureum* is most probably due to an intraspecific polymorphism for a paracentric inversion. Therefore, in spite of the karyological, morphological, and ecological similarities between both *Acrostichum* species, there was no morphological or meiotic evidence of interspecific hybridization, as was reported in specimens from the Dominican Republic by López (1978).

Our data suggest that cytogenetic differentiation between *A. danaeifolium*

and *A. aureum* is limited to very small variations in chromosome morphology and structure. Considering that these two species are sympatric throughout a wide geographical region, occupy narrow ecological niches, and probably have a long history of reproductive isolation it is surprising that there are so few cytological differences between them. Probably, the reproductive isolation is based on genic rather than chromosomic barriers and the karyotypic orthoselection, common in many ferns, has conserved the basic karyotype in both species, even at the level of the distribution of heterochromatin and rDNA sites.

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